CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals, antibodies and instruments used in this study are shown in Appendix A, B and C

2.2 Large scale production and purification of monoclonal antibody (mAbs) MT3 and COSA2A

The mAb MT3 was produced previously in our laboratory by immunoprecipitation based immunization technique. By using CD3 mAb (clone OKT3) coated beads, CD3 complexes were precipitated from peripheral blood mononuclear cells (PBMCs) lysate. The CD3-immunoprecipiated beads were then used as the immunogen for mouse immunization. After hybridoma production, a mAb named MT3 was generated (unpublished observation). In addition, by immunization of monkey kidney COS7 cells, a mAb named COSA2A was generated (unpublished observation). Plasmid encoding CD4 molecule were transfected into COS cells for expression of CD4 molecules on cell surface membrane. The CD4 expressing COS cells were then used as immunogen for mouse immunization. In preliminary studies, we found that both mAb, MT3 and COSA2A, were reacted to sub-population of lymphocytes. Thus, both mAbs were of interested and selected for further investigations, in this study.

2.2.1 Production of ascitic fluid

Hybridoma clones MT3 and COSA2A were generated in our laboratory. To produce large amount of purified mAbs, ascitic fluids containing the mAbs were first produced. Hybridoma clones MT3 and COSA2A were cultured in Iscove's Modified Dulbeco's medium (IMDM) containing 10% heat inactivated fetal calf serum (FCS), gentamycin 40 μg/ml and fungizone 5 mg/ml (10% FCS-IMDM) at 37°C in 5% CO_2 incubator. After the hybridomas cells growing up, cells were collected and washed with phosphate buffer saline (PBS) pH 7.2 for three times by centrifugation at 1,500 g for 5 minutes. The hybridomas were further used for induction of ascitic fluids.

To produce ascitic fluid, mouse was intraperitoneally injected with 0.5 ml of pristine (Tetrametyl-pentadecane). After 1 week of pristine injection, $1x10^6$ hybridoma cells were injected into mouse peritoneal cavity. One to two weeks after hybridoma inoculation, the ascitic fluid was drawn from the induced mouse and centrifuged at 14,000 g, 4°C for 30 minutes to collect the supernatant and stored at 20°C. The indirect immunofluorescent staining was used for testing the activity of monoclonal antibodies (mAb) in all obtained ascitic fluids.

2.2.2 Purification of monoclonal antibodies by affinity chromatography

As both mAbs MT3 and COSA2A were IgM isotype, Hitrap™ IgM column was used for purification of these mAbs. Ascitic fluids were clarified by centrifugation at 14,000 g, 4°C for 30 minutes. The clarified ascitic fluids were diluted with 4X binding buffer (20 mM sodium phosphate, 0.8 M $(NH_4)_2SO_4$, pH 7.5) and were then applied into the Hitrap™ IgM column. The mAbs were eluted from the

column by eluting buffer (20 mM Sodium phosphate buffer pH 7.5). The purified mAb fractions were collected by AKTA prime fraction collector. The obtained mAbs were dialyzed against PBS at 4°C for overnight and determined the protein concentration by measured absorbance at 280 nm. The purified mAbs were stored at -20°C.

The activity and the purity of the purified mAbs were further determined by indirect immunofluorescence staining and SDS-PAGE, respectively.

2.3 SDS-PAGE for determination of the purity of monoclonal antibodies

The purity of the purified mAbs was determined by SDS-PAGE. Briefly, 10 µg of purified mAbs were mixed with reducing and non-reducing buffer and then loaded into each lane of 12.5% SDS-polyacrylamide gel. The electrophoresis was performed at 120 V for 2 hr. The heavy chain and light chain of mAbs were migrated according to their molecular weight. Proteins on the separating gel were visualized by staining with PAGE Blue for overnight. After that the gel was destained with dH_2O.

2.4 Cellular distribution of the molecules recognized by monoclonal antibodies MT3 and COSA2A

2.4.1 Sample preparation

2.4.1.1 Peripheral blood cells

2.4.1.1.1 Peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from EDTA blood by Ficoll-hypaque density gradient centrifugation. Briefly, 10 ml of EDTA blood were diluted with 10 ml PBS.

Then, 20 ml diluted blood were overlaid on 10 ml of Ficoll-hypaque solution and centrifuged at 400 g, 25°C for 30 minutes. The PBMC fraction was collected and washed with PBS for 3 times. The cells were adjusted to 1×10^7 cells/ml in 1% bovine serum albumin in PBS containing 0.02% sodium azide (1% BSA-PBS-0.02% NaN_3).

2.4.1.1.2 Granulocytes

Granulocytes were isolated from EDTA blood by 6% dextran sedimentation. Briefly, 10 ml of EDTA blood were diluted with 10 ml PBS. The diluted blood was added into 8 ml of 6% dextran in PBS and mixed well. Then, the mixture was incubated at 37°C and sit at 45° for 30 minutes. The white blood cell rich fraction was collected and centrifuged at 1,700 g, 25°C for 10 minutes. The contaminated erythrocytes were lysed by adding 10 ml of hypotonic ammonium chloride solution and stood for 5 minutes. The obtained leukocytes were washed with PBS for 3 times and adjusted to 1×10^7 cells/ml in 1% BSA-PBS-0.02% NaN_3

2.4.1.1.3 Erythrocytes

EDTA blood were washed with PBS for 3 times. The cells were then adjusted to 0.3% cells with 1% BSA-PBS-0.02% NaN_3.

2.4.1.2 Hematopoietic cell lines

The hematopoietic cell lines used in this study was shown in Table 2.1. All cell lines were cultured in RPMI-1640 containing 10% heat inactivated fetal calf serum (FCS), gentamycin 40 µg/ml and fungizone 5 mg/ml (10% FCS-RPMI) at 37°C in 5% CO_2 incubator. For staining, cells were washed with PBS 3 times and re-suspended at 1×10^7 cells with 1% BSA-PBS-NaN_3.

Table 2.1 Hematopoietic cell lines used in this study

Name of cell lines	Type of cell lines
K562	Erytho-myelocytic cell line
U937	Human leukemic monocyte lymphoma cell line (Monocytic cell line)
Daudi	Human Burkitt's lymphoma cell line (B cell line)
Molt4	Human acute lymphoblastic leukemia cell line (T cell line)
SupT1	Human acute lymphoblastic leukemia cell line (T cell line)

2.4.2 Indirect immunofluorescent staining

The expression of molecules recognized by mAb MT3 and COSA2A were assessed by indirect immunofluorescent staining using peripheral blood cells and hematopoietic cell lines. The blood samples were blocked Fc receptor by incubating with 10% human AB serum and incubated on ice for 30 minutes. Fifty microlitters of mAbs MT3 and COSA2A were then added into 50 µl of the blocked cells and incubated on ice for 30 minutes. After the incubation, the samples were washed twice with 1% BSA-PBS-0.02% NaN_3. Afterward, 25 µl of FITC-conjugated $F(ab')_2$ fragment of sheep anti-mouse immunoglobulins antibody were then added and incubated on ice for 30 minutes in dark place. Finally, the samples were washed 3 times with 1% BSA-PBS-0.02% NaN_3 and fixed with 1% para-formaldehyde in PBS (1% paraformaldehyde-PBS). The stained cells were then analyzed by a flow cytometer.

2.5 Analysis of molecules recognized by mAbs MT3 and COSA2A on lymphocyte sub-populations.

Expression of molecules of interested on lymphocyte sub-populations were determined by two-color immunofluorescent analysis. Cell surface molecules of lymphocyte sub-populations were identified by using CD19, CD56, CD3, CD4 and CD8 molecules, which are the markers for B lymphocytes, NK cells, T lymphocytes, $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes, respectively.

2.5.1 Analysis of molecules recognized by mAbs MT3 and COSA2A on B lymphocytes and NK cells

PBMCs (1×10^7 cells/ml) were blocked Fc receptor by incubating with 10% human AB serum and incubated on ice for 30 minutes. Fifty µl of mAbs MT3 or COSA2A were added into 50 µl of the blocked cells and incubated on ice for 30 minutes. The cells were then washed two times with 1% BSA-PBS-0.02% NaN_3 and 25 µl of FITC-conjugated $F(ab')_2$ fragment of sheep anti-mouse immunoglobulins antibody were then added into cell suspension and incubated on ice for 30 minutes in dark place. The cells were then washed with 1% BSA-PBS-0.02% NaN_3 for 3 times, and stained with 25 µl of phycoerythrin (PE) conjugated anti-CD19 or anti-CD56 and incubated on ice for 30 minutes. Finally, the cells were washed with 1% BSA-PBS-0.02% NaN_3 for 3 times, fixed with 1% para-formaldehyde-PBS and were then analyzed by flow cytometry.

2.5.2 Analysis of molecules recognized by mAbs MT3 and COSA2A on T lymphocytes and T lymphocyte subsets

2.5.2.1 T lymphocytes

PBMCs (concentration 1×10^7 cells/ml) were incubated with 10% human AB serum and incubated on ice 30 minutes for blocking the Fc receptors. mAbs MT3 (isotype IgM) or COSA2A (isotype IgM) at the volume of 50 µl were added into 50 µl of PBMCs suspension and incubated on ice for 30 minutes. The cells were washed two times with 1% BSA-PBS-0.02% NaN_3 and were then stained with PE conjugated anti-mouse IgM and FITC conjugated anti-CD3 mAb clone OKT3 (isotype IgG) by

incubation on ice for 30 minutes. Finally, the cells were washed with 1% BSA-PBS-0.02% NaN_3 for 3 times and fixed with 1% para-formaldehyde-PBS and analyzed by flow cytometry.

2.5.2.2 CD4 lymphocytes

PBMCs were incubated with 10% human AB serum for blocking Fc receptors. mAb MT3 (isotype IgM) and anti-CD4 mAb clone MT4/3 (isotype IgG) or mAb COSA2A (isotype IgM) and anti-CD4 mAb clone MT4/3 (isotype IgG) at the volume 50 µl were added into 50 µl of PBMCs suspension and incubated on ice for 30 minutes. The cells were washed twice times with 1% BSA-PBS-0.02% NaN_3, and were stained with PE conjugated anti-mouse IgM and FITC conjugated anti-mouse IgG and incubated on ice for 30 minutes. Finally, the cells were washed with 1% BSA-PBS-0.02% NaN_3 for 3 times. Finally, the cells were fixed with 1% para-formaldehyde-PBS and analyzed by flow cytometry.

2.5.2.3 CD8 lymphocytes

PBMCs were incubated with 10% human AB serum for blocking Fc receptors. mAb MT3 (isotype IgM) and anti-CD8 mAb clone MT8 (isotype IgG) or mAb COSA2A (isotype IgM) and anti-CD8 mAb clone MT8 mAb (istype IgG) at the volume 50 µl were then added into 50 µl of cell suspension and incubated on ice for 30 minutes. The cells were washed twice times with 1% BSA-PBS-0.02% NaN_3, and were stained with PE conjugated anti-mouse IgM and FITC conjugated anti-mouse IgG and incubated on ice for 30 minutes. Finally, the cells were washed with 1%

BSA-PBS-0.02% NaN_3 for 3 times. Finally, the cells were fixed with 1% para-formaldehyde-PBS and analyzed by flow cytometry.

2.6 The molecules recognized by mAbs MT3 and COSA2A on $CD45RO^+$, $CD45RA^+$ and $CD45RB^+$ cells

PBMCs were incubated with 10% human AB serum for blocking their Fc receptors. mAbs MT3 (isotype IgM) or COSA2A (isotype IgM) at the volume 50 µl were then added into 50 µl of cell suspension and incubated on ice for 30 minutes. The cells were washed two times with 1% BSA-PBS-0.02% NaN_3, and were then stained PE conjugated anti-mouse IgM and incubated on ice for 30 minutes. The cells were washed twice with 1% BSA-PBS-0.02% NaN_3, and were stained with FITC conjugated anti-CD45RA mAb clone MEM93 or anti-CD45RB mAb clone MEM55 or anti-CD45RO mAb clone UCHL-1 and incubated on ice for 30 minutes. Finally, the cells were washed with 1% BSA-PBS-0.02% NaN_3 for 3 times and fixed with 1% para-formaldehyde-PBS. Cell surface fluorescence was analyzed by flow cytometry.

2.7 SDS-PAGE and Western immunoblotting

2.7.1 Cell lysate preparation

PBMCs were isolated from ACD blood by Ficoll-hypaque density gradient centrifugation (as described in 2.4.1.1.1). Cells were then adjusted to final concentration of 1×10^8 cells in 1 ml of 1% NP-40 lysis buffer (Tris lysis buffer; 50 mM Tris-HCl pH 8.2, 100 mM NaCl, 2 mM EDTA, 0.02% NaN_3, containing 1% NP-40 as detergent and protease inhibitor (1mM phenylmthyl-sulphonylfluoride (PMSF),

5 mM iodoacetamide, 10 ug/ ml aprotinin)) and incubated on ice for 30 minutes. Finally, cell lysates were collected by centrifugation at 12,000 g, 4°C for 30 minutes.

2.7.2 SDS-PAGE and Western immunoblotting

The cell lysates were added with an equal volume of 2X non-reducing (see Appenix C) or reducing buffer (see Appendix C) and boiled for 5 minutes. The cell lysates were then separated by SDS-PAGE using 10% separating gel and 4% stacking gel and subsequently transferred to a nitrocellulose membrane by semi-dry electrophoretic blotting system at 40 mA for 2 hours.

The membranes were blocked with 5% skim milk in PBS at room temperature for 1 hour and were then rinsed twice with PBS and incubated with mAbs MT3 or COSA2A at room temperature for 1 hour. After the incubation, the membrane were rinsed with washing buffer (PBS containing 0.1%Tween 20) for 5 times and incubated with horseradish peroxidase (HRP) conjugated anti-mouse immunoglobulins in PBS containing 1% skim milk at room temperature for 1 hour. The membranes were then washed 3 times with washing buffer and two times with PBS. The protein bands were visualized by the chemiluminescence detection system.

2.7.3 The chemiluminescence detection system

The membranes were incubated with peroxide-luminol/enhancer solution for 5 minutes at room temperature. Then, the membranes were wrapped with enwrap and exposed with a light sensitive clear blue X-ray film (CL-X Posure Film). Finally, the films were developed with Kodak GBX solution.

2.8 Immunoprecipitation

2.8.1 Biotinylation and preparation of cell lysates

PBMCs were isolated from ACD blood by Ficoll-hypaque density gradient centrifugation (see 2.4.1.1.1). Cells were then adjusted to final concentration of 2 x 10^7 cells in 1 ml of 5 mM Sulfo-NHS-LC-biotin in PBS and incubated on ice for 1 hour. The biotinylation reaction was stopped by washing with 1 mM glycine in PBS and then twice with cold-PBS. The biotinylated cells at 5×10^7 cells were lysed with 1% NP-40 lysis buffer and incubated on ice for 30 minutes. Finally, cell lysates were collected by centrifugation at 12,000 g, 4°C for 30 minutes.

2.8.2 Preclearing of biotinylated cell lysates

Five hundred microliters of protein G sepharose beads were coated with 200 µl of normal mouse serum. Following the rotation at 25°C for 4 hours, the beads were washed with PBS for 5 times. Then, 1 ml of biotinylated cell lysates were added into the serum coated beads and rotated at 4°C for overnight. The precleared biotinylated cell lysates were collected by centrifugation at 12,000 g, 4°C for 5 minutes.

2.8.3 Coupling of monoclonal antibodies on protein G Sepharose beads

Two hundred microliters of the protein G Sepharose beads were coated with 150 µg of goat anti-mouse IgM antibodies. Following the rotation at room temperature for 4 hours, the beads were washed with PBS for 5 times. Then, 50 □l of

the coated beads were incubated with 50 µg of mAb MT3 and rotated at 4°C, for overnight.

2.8.4 Immunoprecipitation

One milliliter of precleared biotinylated cell lysates were added to mAb MT3 - coupling beads and rotated at 4°C overnight. After ten times washing with lysis buffer, the protein complexes were dissociated from the beads by boiling in 50 µl of non-reducing or reducing SDS-PAGE sample buffer for 5 minutes. The precipitated proteins were separated by SDS-PAGE using 10% separating gel and 4% stacking gel at 120 volts (constant volt) and subsequently transferred to a nitrocellulose membrane by semi-dry electrophoretic blotting system at 40 mA (constant ampere) for 2 hours. The membranes were blocked with 5% BSA in PBS at room temperature for 1 hour.

Then, the blocked membranes were rinsed twice with PBS and incubated with horseradish peroxidase (HRP) conjugated streptavidin (1:30,000) in PBS containing 1 % BSA-PBS at room temperature for 1 hour. Thereafter, the membranes were washed 3 times with washing buffer and twice with PBS. The proteins were visualized by the chemiluminescence detection system (see 2.7.3).

2.9 Surface expression of MT3 molecules and COSA2A molecules on mitogen-activated PBMCs

PBMCs at a concentration of 1×10^6 cells/ml were isolated from heparinized blood by ficoll-hypaque gradient centrifugation. PBMCs were cultured with 10% FCS-RPMI in the present or absent of PHA at final concentration of 2 µg/ml in 24-

well tissue culture plates at 37°C in a 5% CO_2 incubator. At the first and the third day of the cultivation, cells were harvested and examined for the expression of MT3 and COSA2A molecules by indirect immunofluorescence staining.

2.10 Proliferation assay

2.10.1 Immobilization of anti-CD3 mAb

Anti-CD3 mAb clone OKT3 at a concentration of 60 ng/ml in sterile PBS were prepared. To immobilize the mAb OKT3 on culture plate, 100 µl of mAb OKT3 were dispensed to each well of the 96-well tissue culture plates. For the unstimulated control, wells were added with 100 µl of sterile PBS. Then, the plates were incubated at 4°C overnight.

2.10.2 Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling of PBMCs

PBMCs were isolated from heparinized blood by ficoll-hypaque gradient centrifugation with sterile technique and adjusted to the concentration of 1×10^7 cells/ml in PBS. The CFSE were diluted in sterile PBS to give final concentration of 100 µM from the stock of 5 mM. Five microliters of the diluted CFSE were added into PBMCs 1×10^7 cells/ml to give final concentration of 0.5 µM and mixed by gently pipetting cells up and down. The PBMCs were then incubated at 37°C in water-bath for 10 minutes. After incubation, 10 ml of cold 10% FCS RPMI were added and immediately centrifuged at 1,500 g, 4°C for 10 minutes. The cells were then washed with 10% FCS-RPMI for 2 times. Finally, the cells were counted and adjust the

concentration to 1×10^6 cells/ml in 10% FCS- RPMI. The CFSE labeled PBMCs were checked with flow cytometric analysis.

2.10.3 Proliferation assay

Before adding the CFSE labeled PBMCs, mAb OKT3 immobilized plate (see 2.10.1) was washed with 200 µl/well of PBS and blocked each well with 100 µl of 1% BSA-PBS for 1 hour. After the incubation, the OKT3 immobilized plate was washed twice with 200 µl/well of PBS. Then, mAbs MT3 or COSA2A or media control and isotype match control antibody and inhibitory control antibody at a concentration of 100 µg/ml in 10% FCS RPMI were added into each well. Then, the CFSE labeled PBMCs (see 2.10.2) were added into each well. The mixture was cultured at 37°C in a 5% CO_2 incubator. At the third, fifth and seventh day of cultivations, the cells were harvested and assessed by flow cytometry.